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Fate of fluorescent microspheres in developing *Ictalurus punctatus* following prolonged immersion

Gavin W. Glenney [a,1], Lora Petrie-Hanson [b,*]

[a] *U.S.D.A. National Center for Cool and Cold Water Aquaculture, 11861 Leetown Road, Kearneysville, WV 25430, United States*
[b] *College of Veterinary Medicine, Mississippi State University, Starkville, P.O. Box 6100, MS 39762-6100, United States*

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Abstract

Particulate antigen uptake by the mucosa of developing channel catfish was determined by immersing larvae and fry [2-day post-hatch (dph), 1-, 2-, 3-, 4-, and 8-week post-hatch (wph)] to two forms of fluorescent microspheres (FMS): blue FMS were carboxylated, and green FMS were coated via conjugation with a crude extract of *Edwardsiella ictaluri* outer membrane protein (OMP). Phagocytosis, destination, and clearance appeared similar for the two types of FMS used. In the older age classes, primary uptake was observed in epithelial cells of the torso, fins, nares and to a lesser extent the gills. Fluorescent microspheres were less frequently observed within mononuclear phagocytes in the epidermis, dermis and underlying connective tissue of the tissue mentioned above. Limited FMS trafficking was observed from 4- to 24-h post-immersion (hpi). Significantly higher numbers of FMS (blue and green)/mm^3 of tissue were observed in the posterior kidney of the 4- and 8-wph age classes and in the anterior kidney and spleen of the 8-wph age class when compared to younger age classes ($p < 0.05$). Significantly higher FMS (blue and green)/mm^3 of tissue were observed in the posterior kidney of 4- and 8-wph fish when compared to all other organs ($p < 0.05$). The present study indicates that FMS uptake increases with age in channel catfish. The younger age classes may possess an increased ability to exclude particulate antigen, or lack the specific mechanisms that needed to take up particulates in the form of FMS.

Keywords: Particulate uptake; Innate immunity; Immersion; Fluorescent microspheres; *Ictalurus punctatus*; Clearance

1. Introduction

Stocking densities and feeding rates associated with intensive aquaculture lead to favourable conditions for aquatic pathogens. These pathogens can cause extensive economical hardship, thus the desire to administer effective vaccines has increased [1]. To maximise protection, culturists want to administer vaccines as early in the life cycle as possible. Vaccination via immersion allows culturists to inexpensively vaccinate large numbers of small fish while reducing the stress associated with handling. However, acquired immunocompetence observed in adult fish is not achieved for several weeks to months post-hatching [2−6]. To minimise costs and maximise vaccine effectiveness without inducing

* Corresponding author. Tel.: +1 662 325 1291; fax: +1 662 325 1031.
E-mail addresses: gglenney@ncccwa.ars.usda.gov (G.W. Glenney), lora@cvm.msstate.edu (L. Petrie-Hanson).
[1] Tel.: +1 304 724 8340x2186; fax: +1 304 725 0351.

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immunological tolerance or suppression, finding the appropriate age to vaccinate individual fish species is essential to aquaculture industries.

In attempts to develop cost effective subunit vaccines, various outer membrane proteins of *Edwardsiella ictaluri* have been found to be immunogenic to channel catfish [7,8]. Fluorescent microspheres covalently conjugated to a crude extract of *E. ictaluri* outer membrane proteins were used in the present study in conjunction with plain carboxylated FMS to evaluate immunogenic differences. This study addressed when and where particulate antigen (FMS) sequestering and trafficking occurs in the mucosa of developing channel catfish. Additionally, it was determined if these processes are different in larvae compared to juvenile catfish.

2. Materials and methods

2.1. Channel catfish

Channel catfish were spawned in one-tenth acre ponds at the Animal Research Center, Aquaculture Unit, Mississippi State University. Egg masses were collected and transferred to the Aquatic Medicine Research Laboratory at the College of Veterinary Medicine, Mississippi State University. Egg masses were disinfected with 4.0 ppm potassium permanganate for 20 min, and then temperature acclimated. The eggs were then placed in a solution of 1.5 ppt sodium sulphite for 8–10 min to dissolve the gelatinous matrix, rinsed, and treated with 150 ppm formalin for 10 min. The eggs were then placed in 7-l upwelling hatching jars and incubated at 25–28.0 °C until hatched. Upon swim-up, fry were fed with 50.0% protein salmon/trout starter (Neilson and Sons – Logan, Utah) at 15–20.0% body weight per day at 4–8-day post-hatch. The developing fry were used at specific ages for the experiments described below.

2.2. Fluorescent microsphere immersions

Two forms of carboxylate-modified fluorescent microspheres (FMS), 1.0-μm polystyrene (Polysciences, Inc., USA), were used as particulate antigens in a series of immersion exposures. The first form was a carboxylated FMS (blue) (Polysciences, USA). The second form consisted of FMS (green) covalently conjugated to a crude extract of *E. ictaluri* outer membrane protein (OMP). To isolate OMP, *E. ictaluri* was grown overnight, heat inactivated for 60 min at 60 °C, and washed in PBS. The bacteria were then centrifuged at 1500 × g for 20 min. The supernatant was discarded and the pellet was resuspended in PBS and centrifuged at 1500 × g for 20 min. The pellet was then mixed with a 0.5% deoxycholic acid buffer and incubated for 60 min at 33 °C, and re-centrifuged at 1500 × g for 20 min. The supernatant was used for OMP conjugation.

The conjugation process was conducted with a commercial conjugation kit (Polysciences, Inc., USA, cat#21758). Fluorescent microspheres were washed (2×) in 0.1 M carbonate buffer. Three more washes in 0.02 M phosphate buffer followed. The FMS were suspended in this same phosphate buffer with a 2% carbodiimide solution. The FMS were then mixed for 3 h at room temperature. The FMS were then pelleted and washed (2×) in a 0.02-M phosphate buffer. The FMS pellet was resuspended in a 0.2-M borate buffer and 400 μg of outer membrane protein was added. The solution was gently mixed overnight. The extent of the protein conjugation was confirmed by measuring protein levels in the discarded supernatant. Non-specific protein binding sites were blocked with bovine serum albumin (BSA) and the FMS were held in storage buffer. To confirm protein presence on FMS, sodium dodecyl sulphate polyacrylamide gel electrophoresis was conducted on the supernatant after boiling washed FMS. The gel was then stained with Coomassie Brilliant Blue R-250 staining.

Immersions were conducted for 24 h, using an equal mixture of OMP conjugated FMS (green) and carboxylated FMS (blue) at a concentration of 1×10^7 microspheres ml^{-1} [9–11]. Control fish were held for 24 h without FMS immersion. Six age classes of developing channel catfish were immersed at either 2-day post-hatch (dph) 1-, 2-, 3-, 4-, and 8-wph at average weights of 23.0, 35.0, 72.0, 118.0, 152.0, and 1880.0 mg, respectively. After immersion, controls and exposed fish were placed in a de-chlorinated city water flow-through system maintained at 24–27.0 °C until sampling.

2.3. Sampling, histological and staining procedures

Three to five fish per treatment were sampled at 0-, 4-, and 24-h post-immersion (hpi), and 7, 14, and 21-day post-immersion (dpi). All sampled fish were euthanised with 350 ppm Finquel (Argent Chemical Laboratories – Redmond, Washington), placed in tissue embedding media (Triangle Biomedical Sciences, TBS™–Tissue Freezing Media™), snap frozen in liquid nitrogen and stored at −80.0 °C until used. Due to size constraints, only selected organs and tissue (posterior kidney, anterior kidney, spleen, heart, and gill) of the 8-wph age class were frozen. Frozen samples of whole fish and organs were serially sectioned at 8–12 μm on a cryostat (Triangle Biomedical Sciences, Minotome Plus™, USA) and placed on poly L-lysine coated slides (Sigma Poly-Prep™). Thirty to forty sections were made for each fish. Two fixatives were used depending on the stain to be used. A glutaraldehyde fixative solution (25.0 ml acetone and 75.0 ml glutaraldehyde solution – Sigma) was used for Gill No. 3-haematoxylin (6.0 g L^{-1}, sodium iodate) and Sudan Black B (0.18% w/v, in 69.0% ethanol and containing phosphate-buffered phenol – Sigma Diagnostics). A second glutaraldehyde solution (9.0 ml glutaraldehyde – 25.0% in water (Sigma), 21.0 ml deionised water, and 45.0 ml acetone) was used to fix slides stained with enzyme substrates (Lymphocyte Enzyme Kit – Sigma) optimised for channel catfish [12].

2.4. Fluorescent microsphere counts

Slides were viewed with a fluorescent microscope (Olympus America Inc., Olympus BX60, USA) at optical wavelengths between 450 and 80 nm. Initially, 16 sections (8 – haematoxylin, 8 – Sudan Black B) of each fish were examined for the presence of FMS associated with cutaneous, gill, and gut mucosa. Fluorescent microspheres were counted and total numbers were determined by location. Additionally, three representative sections (haematoxylin stained) of each organ (posterior kidney, anterior kidney, spleen, and heart) were viewed and FMS/mm^3 of tissue were determined. Additionally, cell populations closely associated with FMS were identified.

2.5. Statistical analysis

Statistical comparisons of mean FMS/mm^3 of tissue between organs, sample times, and age groups were conducted using Log_{10} transformed data. Transformed data were analysed by analysis of variance (ANOVA) using SAS (Statistical Analysis Software Institute Inc., Cary, N.C., USA) procedures, General linear model and Mixed. $p < 0.05$ was accepted as significant. Data from each FMS type were analysed separately.

3. Results

3.1. Fluorescent microspheres

Both FMS types were observed in the same types of phagocytes (often in the same cells), trafficked to the same locations, and cleared at the same rates. No statistically significant difference was found between mean 'green' and 'blue' FMS/mm^3 of tissue ($p > 0.05$); so from here forward, 'FMS' will be used as a inclusive term, unless otherwise specified. Significant differences in FMS/mm^3 of tissue were demonstrated by ANOVA between age classes and organs for both types of FMS ($p < 0.05$).

3.2. Uptake and clearance

The majority of the FMS uptake occurred in the tissues of the external epithelium and increased with age (Table 1). Primary uptake sites were the head, torso, fins, nares, and to a lesser extent, the gills. Most FMS were phagocytosed by negative enzyme staining epithelial cells. Fluorescent microspheres were less frequently observed within acid phosphatase (AP) and α-naphthyl butyrate esterase (NBE) positive mononuclear phagocytes (MPs) in the epidermis and connective tissue of the locations mentioned above. Occasionally, FMS appeared to be within AP and NBE positive MPs at the epithelial-water interface of the secondary and primary gill lamellae. Fluorescent microspheres were rarely observed free or within MPs in the posterior kidney vasculature from 4- to 24-hpi. Fluorescent microspheres were also found in intestinal epithelium, lamina propria, and muscularis, but in low numbers.

Fluorescent microsphere concentrations peaked from 24-hpi to 7-dpi in the anterior and posterior kidney and spleen of the 3-, 4-, and 8-wph age classes (Table 2).

3.3. Age specific response

3.3.1. 2-Day post-hatch

Very few FMS were observed in any tissue examined of this age class (Tables 1 and 2).

Table 1
Chronological presentation of total fluorescent microsphere numbers in developing channel catfish mucosa by sample time and tissue location

Age	Number of fish sampled	Time post-immersion	Free along surface of gill and skin, or in mucus	Gill epi.	Free w/in nares chamber	Nares epi.	Body epi.	Fin epi. and conn. tissue	Gut lumen			Air bladder lumen
									Oesophagus	Stomach	Intestines	
2-dph	4	0 h	X ¾			¼	½		¼			
	4	4 h	¼				¼					
	4	24 h	¾									
	5	7 d	⅕				⅕				⅕	
	4	14 d										
	0	21 d										
1-wph	4	0 h	X ½	½	½		¾	X	X ¾	XXXXX	XXXXX	X
	4	4 h	X ¼	¼	¼	½	X		¼	XXXX ½	XXXX ½	½
	5	24 h	⅕		⅕		⅘	⅘		⅕ (epi.)	⅘	
	4	7 d		¼			¼	¼			¼	
	3	14 d	⅔		⅓	⅔	X				⅓ (l.p.)	
	3	21 d		⅓			X	⅓				
2-wph	5	0 h	X ⅕	X	⅕	⅘	X ⅗	X ⅕	X ⅗	XXXX ⅕	XXXXX	⅖
	4	4 h	¼	½		¾	X	X		X ½	XXXX	
	4	24 h		¼	½	¾	¾	X ½		X	¾, ½ (epi.)	
	4	7 d		¼	½	½	¾	½			¼, ½ (epi.)	
	3	14 d		⅔		⅔	⅔	X				
	3	21 d				⅔	X					⅓
3-wph	4	0 h	XXXX ½	XX ½	X ½	½	X ¾	X	XX ¼	XXXXX	XXXXX	
	4	4 h	XXXX ¼	XX	¼	¾	X ¼	¾	¾	XX ½	XXXX ¼	
	4	24 h	XX ¼	X		X	X	X ¼			XX ¼	¼
	4	7 d		¾		X	¾	¾			½, ¼ (mus.)	
	3	14 d		⅓		⅔	⅔	⅔				⅓
	3	21 d				⅔	⅔			⅓ (l.p.)		
4-wph	4	0 h	X ½	X ¾	X ¼	X ½	X ½	X	½	XXXX	XXXX ¾	
	4	4 h	¾	¾		X ½	X ¼	X	¼, ¼ (epi.)	XX	XXXX ¼	¾
	4	24 h		¼	¼	X ½	X	X ½		½		
	4	7 d		¼		X	¾	½	¼ (epi.)	¼ (mus.), ¼ (epi.)	¼, ½ (epi.)	
	3	14 d				⅔	⅔	⅓		⅓ (mus.)	⅓ (l.p.)	
	3	21 d				⅓	⅓					
8-wph	4	0 h	XXX ½	XX	–	–	–	–	–	–	–	–
	4	4 h	X ¼	X ½	–	–	–	–	–	–	–	–
	4	24 h		X ¼	–	–	–	–	–	–	–	–
	4	7 d		X ¼	–	–	–	–	–	–	–	–
	4	14 d		¼	–	–	–	–	–	–	–	–
	4	21 d		¼	–	–	–	–	–	–	–	–

Fluorescent microsphere numbers: fractions = number of fish out of total = X, <10 = X, 10–60 = XX, 60–250 = XXX, 250–1000 = XXXX and >1000 = XXXXX. dph = Days post-hatch, wph = weeks post-hatch, h = hours, d = days, epi. = epithelium, conn. = connective tissue, l.p. = lamina propria, and mus. = muscularis.

Table 2
Chronological presentation of mean fluorescent microspheres/mm^3 (±SE) of developing channel catfish tissues

Age	Time post-immersion	Blue				Green (OMP)			
		Anterior kidney	Heart	Posterior kidney	Spleen	Anterior kidney	Heart	Posterior kidney	Spleen
2-dph	0 h–14 d	0	0	0	0	0	0	0	0
1-wph	0 h–14 d	0	0	0	0	0	0	0	0
	21 d	0	0	0	26 ± 26	0	0	6 ± 6	0
2-wph	0 h	0	0	0	0	0	0	0	0
	4 h	0	0	0	0	0	0	14 ± 14	0
	1 d–21 d	0	0	0	0	0	0	0	0
3-wph	0 h–4 h	0	0	0	0	0	0	0	0
	1 d	0	0	37 ± 37	87 ± 87	0	0	40 ± 27	0
	7 d	0	0	0	35 ± 35	0	0	9 ± 9	0
	14 d	0	0	5 ± 5	0	93 ± 93	0	0	0
	21 d	0	0	0	0	0	0	0	0
4-wph	0 h	0	0	37 ± 24	58 ± 58	0	0	0	0
	4 h	0	0	4 ± 4	0	0	0	0	0
	1 d	0	0	139 ± 103	56 ± 56	87 ± 87	0	202 ± 166	0
	7 d	0	0	0	0	0	0	0	0
	14 d	0	0	0	0	3 ± 3	0	0	0
	21 d	0	0	4 ± 4	0	0	0	18 ± 9	0
8-wph	0 h	2 ± 2	0	16 ± 6	0	6 ± 4	0	13 ± 8	12 ± 12
	4 h	5 ± 3	0	6 ± 2	0	2 ± 2	0	7 ± 6	11 ± 11
	1 d	7 ± 4	0	8 ± 6	13 ± 8	5 ± 5	0	3 ± 2	6 ± 6
	7 d	0	0	11 ± 6	7 ± 7	6 ± 3	0	13 ± 7	7 ± 7
	14 d	4 ± 4	0	2 ± 1	5 ± 5	4 ± 4	0	4 ± 3	8 ± 5
	21 d	2 ± 2	0	4 ± 2	0	6 ± 6	0	4 ± 3	0

Counts were categorised by fluorescent microsphere type and averaged by sample time within each age class. dph = Days post-hatch, wph = weeks post-hatch, h = hours, and d = days.

3.3.2. 1-Week post-hatch

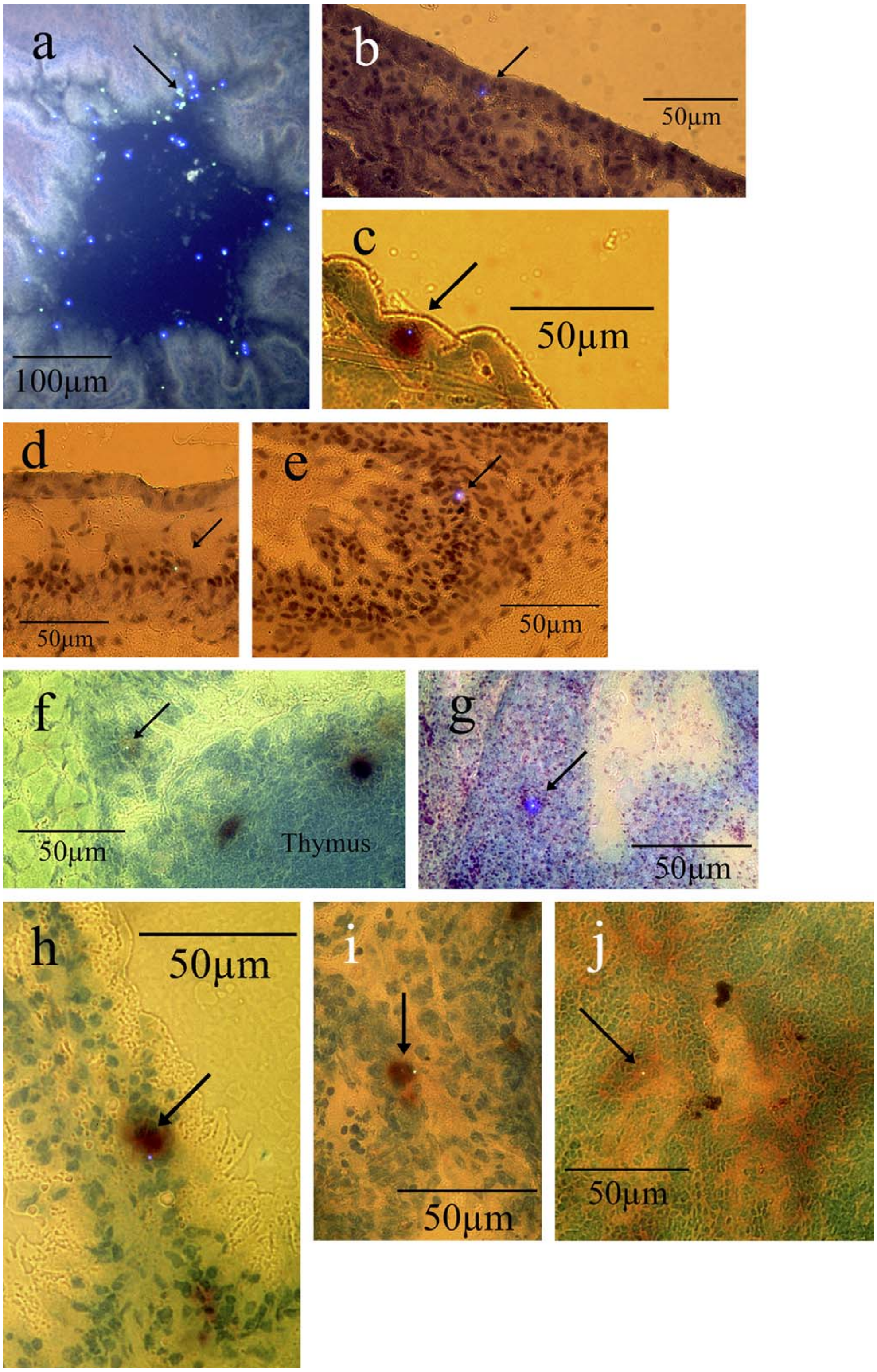
Unlike the 2-dph age class, thousands of FMS were ingested and observed from 0- to 24-hpi in the stomach and intestinal lumen of this age class and all following age classes (Fig. 1a). Fluorescent microspheres were observed in gill and nares epithelial cells and the lumen of the air bladder adjacent to the epithelial surface. Epithelial cells of the torso contained FMS in all PI sample times (Fig. 1b). Occasionally, FMS were observed within AP positive MPs of the abdominal and pectoral fin epithelium. Fluorescent microspheres were not observed in the posterior kidney and spleen until 21-dpi. Limited numbers of FMS were found at earlier PI sample times within AP and NBE positive MPs randomly distributed throughout the posterior kidney, while a few FMS were located next to NBE positive melanomacrophage centers (MMC) in the spleen. No significant differences were observed in FMS/mm^3 between organs in this age class (Fig. 2).

3.3.3. 2-Week post-hatch

When compared to earlier ages, epithelial cells of the torso, fins, nares, and gills demonstrated an increased uptake of FMS. Only occasional AP and NBE positive MPs contained FMS (Fig. 1c). From 4- to 24-hpi, FMS were randomly distributed in the posterior kidney, but were found in interrenal tissue negative for enzyme stains. Fluorescent microspheres were rarely found in the epithelium of the gut and anal vent. No significant differences were observed in FMS concentrations between organs of this age class (Fig. 2).

3.3.4. 3-Week post-hatch

When compared to the 2-dph, 1- and 2-wph age classes, a marked increase in FMS uptake was observed in the epithelial cells of the torso, fins, nares, and especially the gills (Fig. 1d, e). Fluorescent microspheres were more frequently observed within AP and NBE positive MPs in the peripheral mucosa and underlying connective tissue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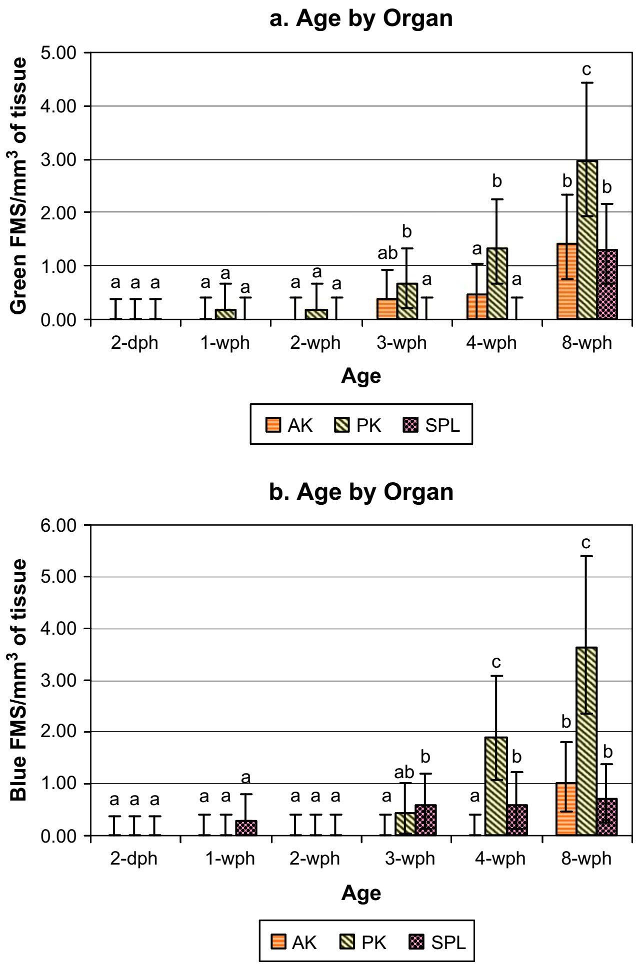

Fig. 2. Concentration of fluorescent microspheres (FMS) observed in the anterior kidney, posterior kidney, and spleen compared by age class over a 21-day study period of fish immersed in (a) green-*Edwardsiella ictaluri* conjugated FMS; (b) blue carboxylated FMS. Values are geometric means ± 95% confidence limits averaged across all sample times by organ ($n = 21-24$). Bars labelled by different letters represent significant differences by ANOVA ($p < 0.05$).

(Fig. 1f). This was primarily at the fin bases, nares and gills. In addition, FMS were found more often in AP and NBE positive MPs of the posterior kidney and in and along the perimeters of the β-glucuronidase (BG) positive periarteriolar lymphoid sheathing (PALS) of the spleen. Fluorescent microsphere concentrations peaked in the posterior kidney and spleen from 24-hpi to 14-dpi, and were absent in these organs by 21-dpi. This was the first age class in which FMS were observed in the negative staining interstitium of the anterior kidney.

Significantly higher FMS/mm^3 were observed in the posterior kidney than that in the spleen of 3-wph fish for green FMS, and to the anterior kidney for blue FMS (Fig. 2).

Fig. 1. Histological sections demonstrating fluorescent microspheres (FMS) (arrows) in developing channel catfish tissues: (a) 1-wph, FMS within the stomach lumen at 4-h post-immersion (hpi) (β-glucuronidase); (b) 1-wph, FMS within ventral epithelium at 0-hpi (haematoxylin (Haem)); (c) 2-wph, FMS within α-naphthyl butyrate esterase (NBE) positive mononuclear phagocyte (MP) in ventral epithelium at 21-day post-immersion (dpi); (d) 3-wph, FMS below alarm cells at dermal and epidermal interface at 7-dpi (Haem); (e) 3-wph, FMS within nares epithelium at 7-dpi (Haem); (f) 3-wph, FMS within NBE positive MP in epithelial tissue along thymus at 24-hpi; (g) 4-wph, FMS within acid phosphatase (AP) positive phagocyte in lateral epithelial tissue at 7 days post-immersion (dpi); (h) 4-wph, FMS within NBE positive mononuclear phagocyte (MP) in nares epithelium at 7-dpi; (i) 8-wph, FMS within and along the surface of a NBE positive MP of the gill epithelial tissue and water interface at 24-h post-immersion; and (j) 8-wph, FMS within β-glucuronidase positive periarteriolar lymphoid sheathing at 7-dpi.

3.3.5. 4-Week post-hatch

This was the first age class in which FMS were observed in organs sampled at 0-hpi. The majority of the FMS taken up by the external mucosa were still observed in negative enzyme staining epithelium and underlying connective tissue of the torso, fin bases, nares and gills. This age class exhibited more AP and NBE positive MPs in the peripheral mucosa than that of earlier ages (Fig. 1g, h). As observed at 3-wph, FMS concentrations peaked in the anterior and posterior kidney and spleen at 24-hpi. Approximately half of the FMS were found in AP and NBE positive MPs distributed in the anterior and posterior kidney and in and along the perimeters of splenic BG positive PALS. Fluorescent microspheres were observed more often in or in close association with AP and NBE positive MMCs of the posterior kidney and spleen of the 4- and 8-wph age classes.

A significant increase in FMS concentration was observed in the posterior kidney when compared to 2-dph, 1- and 2-wph fish, and 3-wph fish for blue FMS (Fig. 3). Significantly higher FMS/mm^3 were observed in the posterior kidney when compared to all other organs (Fig. 2).

3.3.6. 8-Week post-hatch

Both FMS types were observed in organs sampled at 0-hpi. This was the first age class to consistently exhibit FMS in a majority of the organs sampled. Fluorescent microspheres were observed within AP and NBE positive MPs of the primary and secondary lamellae epithelium and were occasionally observed at the epithelium-water interface (Fig. 1i). More than half of the FMS in the anterior and posterior kidney were found within AP and NBE positive MPs. These phagocytes were primarily found in the interrenal tissue of the posterior kidney and to a lesser degree next to renal

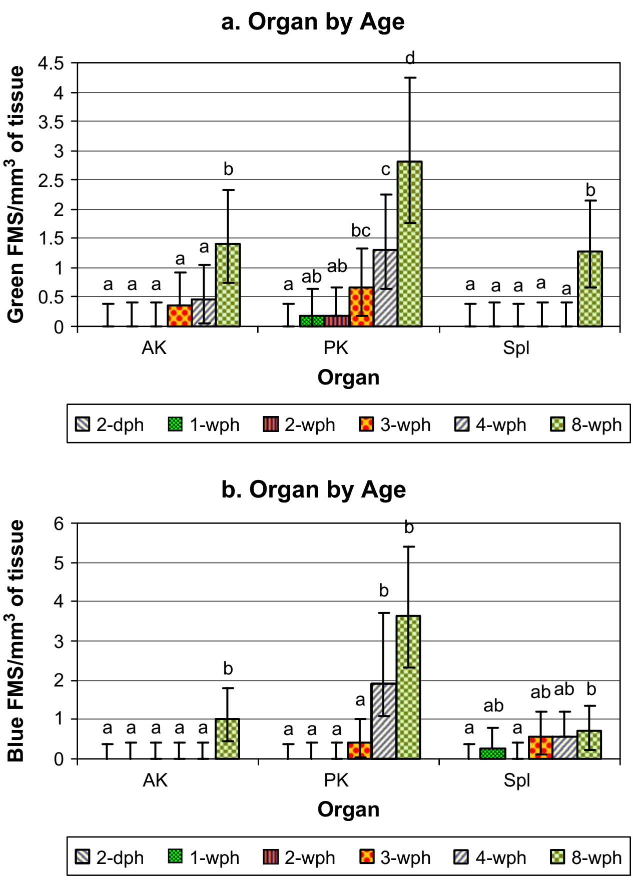

Fig. 3. Concentration of fluorescent microspheres (FMS) observed in the anterior kidney, posterior kidney, and spleen compared by specific age classes over a 21-day study period of fish immersed for 24-h in: (a) green-*Edwardsiella ictaluri* conjugated FMS; (b) blue carboxylated FMS. Values are geometric means ± 95% confidence limits averaged across all sample times by organ ($n = 21-24$). Bars labelled by different letters represent significant differences by ANOVA ($p < 0.05$).

tubule cells. A limited number of FMS were observed within renal tubule cells. Compared to younger age classes, higher concentrations of FMS were observed within AP and NBE positive MPs and MMCs in and along the periphery of BG positive PALS (Fig. 1j).

Significantly higher concentrations of FMS were observed in the majority of the 8-wph age class organs when compared to all younger age classes (Fig. 3). Significantly higher FMS/mm^3 were observed in the posterior kidney when compared to all other organs (Fig. 2).

3.4. Control samples

Control samples across all ages exhibited similarly staining phagocytic populations as FMS immersed fish, with no apparent differences in staining intensity.

4. Discussion

The present results indicate that 2-dph to 2-wph developing channel catfish have an ability to externally exclude particulate antigen in the form of FMS. Similar results were observed for both carboxylated FMS (blue) and FMS (green) *E. ictaluri* OMP conjugated FMS. Histologically, a differential immune response to antigenic proteins was not observed to be elicited. This may indicate that a ''true'' non-specific response was elicited, or a specific response was undetectable by the histological methods used. The response elicited to blue FMS could have been masked by the overall response to the green *E. ictaluri* OMP–FMS.

Initially, the majority of FMS observed were trapped into mucus along the epithelial surfaces of the skin (head and trunk), fins, and most notably in between primary and secondary gill lamellae. This was a clear demonstration of the physical properties of mucus and its role in protecting mucosal surfaces from the external environment.

Channel catfish begin feeding upon swim-up between 5 and 10 days post-hatch, which coincides with the first appearance of FMS in the gastrointestinal tract. In the present study, FMS were not ingested until 1-wph, indicating vaccines that have to be absorbed through the gut may have limited efficacy until after 1-wph. Pappo and Ermak [13] observed the uptake of FMS (600–700 nm) by rabbit follicle-associated epithelial M-cells 10 min after administration. The FMS were phagocytosed and transepithelially transported to Peyer's patch domes. The hydrophobic properties of polystyrene and latex beads have been noted to enable adherence and uptake by mammalian M-cells [14]. Presently, M-cells have not been characterised in channel catfish. Gut epithelial cells were rarely observed to take up FMS in the present study. When FMS uptake was observed, it was most often in the epithelium of the anus. Dalmo et al. [15] observed negative intestinal uptake of orally administered FMS (0.045, 0.134, 0.49, and 3.1 μm) in Atlantic salmon, *Salmo salar*, while Tatner et al. [16] observed no uptake through the gut wall via oral or bath immersion of a formalin killed ^{14}C-labelled *Aeromonas salmonicida* vaccine.

In the present study, epithelial cells appeared to play a major role in antigen uptake. The major uptake sites were the skin (trunk), fins, nares and to a lesser extent the gill epithelium. To our knowledge, this was the first report of FMS uptake by nares epithelium. Fluorescent microspheres were observed to a lesser extent within AP and NBE positive MPs in the epidermis and underlying dermis and connective tissue at the sites mentioned above. These observations indicate that mucosal MPs are instrumental in sequestering and possible trafficking of particulate antigen. A possible explanation for the increase in FMS observed in lymphoid organs with increasing age may be that epithelial cells or migrating professional antigen-presenting cells are functionally mature at the older ages. Mature phagocytes may be actively sampling antigen and presenting it locally or trafficking it to primary lymphoid organs.

In the present study, neutrophils were observed to be associated with MPs containing FMS, but were not observed to be playing a dominant phagocytic role. Goldes et al. [17] observed similar results when they immersed 18-month-old rainbow trout in a kaolin clay–water suspension for up to 32 days. Intracellular clay particles (5 μm) were observed within gill epithelial cells and macrophages underlying the surface epithelium. Neutrophils were not observed to contain particulate material [17].

Mammalian intestinal epithelial cells (IECs) do express MHC II, which can be recognised by T-cell receptors [18]. Channel catfish epithelial cells may have similar properties to mammalian IECs, by expressing non-specific receptors such as CD1, which enables mammalian IECs to present glycolipid and hydrophobic protein antigens of large sizes [18]. Catfish epithelial cells may be functioning in a similar way by taking up particulate antigen and presenting to lymphocytes, or may be functioning like mammalian M-cells by delivering antigen to professional antigen-presenting cells such as peripheral macrophages and or dendritic-like cells.

In the present study, FMS were observed in peripheral tissues (body and nares) for up to 21-dpi. Limited amounts of FMS were trafficked to primary lymphoid organs, thus demonstrating the possibility of peripheral presentation. Moore et al. [9] found similar results. They determined epithelial cells of the skin and gills of rainbow trout (1.5–3.0 g) fingerlings immersed in 1.0 μm BSA-conjugated FMS to be the functional sites of particulate uptake, with the skin (trunk) being the principal site. Fluorescent microspheres were observed to be in surface epithelial cells of the skin and underlying phagocytes. They observed the majority of FMS to remain in the gill and epidermal tissue for up to 24 days post-exposure while minorities of FMS were transported to the spleen and kidney.

Lobb [19] determined that the secretory and systemic immune systems of channel catfish could be differentially stimulated by bath immersion in dinitrophenylated-horse serum albumin. He showed that the majority of the immersed fish responded with a cutaneous antibody response, while the minority exhibited a serum antibody response. Zilberg and Klesius [20] observed mucus Ig levels to be lower and lag 2 weeks behind serum Ig levels following infection with *E. ictaluri*. The results from these two studies support what was observed in the present study as possible peripheral presentation.

A series of FMS immersion studies were conducted with rainbow trout (1.0–5.0 g) immersed in 1.0×10^7 FMS/ml [10,11,21]. As in the present study, FMS clumping in mucus and debris between secondary lamellae of the gills was observed. Investigators found FMS at areas of microscopic injury within and below the migrating epithelial cells present during the wound healing process. They observed the cell membranes of macrophages in superficial layers of the gill epithelium partially exposed to the environment. This was occasionally observed in the present study, but more often FMS were observed within MPs beneath the gill epithelium.

In the present study, the kidney contained the highest concentrations of FMS, while the highest concentrations of FMS tended to be in all organs from 24-hpi to 7-dpi for all ages. Similar results were obtained by Kiryu et al. [11], who observed melanin-laden macrophages to congregate at sites of FMS uptake and phagocytosed microspheres from 8- to 20-dpi. Fluorescent microspheres were also found within MMCs of the kidney and the spleen at this time. They also observed more FMS in the kidney opposed to the spleen; however, they determined that more FMS were present at 20-dpi.

Petrie-Hanson and Ainsworth [6] observed that channel catfish fry vaccinated at 21-dph were the youngest age group to demonstrate a specific antibody response. The results of antigen exclusion or a lack of ability in antigen uptake observed in the present study before 3-wph coincides with these findings. It would be advantageous for developing channel catfish to exclude particulate antigens before a functional acquired response can be elicited. This exclusion or lack of antigen uptake would limit pathogen access and reduce the chances of immunological tolerance or suppression. The present results suggest that FMS conjugated bacterins administered early in development may not gain adequate access to peripheral or primary antigen presenting sites at the levels needed to elicit productive acquired responses. This may not be the case with attenuated live vaccines. However, a reduction in virulence factors may inhibit a live vaccines' ability to penetrate the host's defences and become persistent.

The present results appear similar to what has been found in rainbow trout bath vaccination studies. Tatner and Horne [22] found that fry exposed to *Vibrio anguillarum* via immersion before 6-wph were more resistant, and they attributed this to insufficient numbers of bacteria being able to gain access to the fry. They also found that a period of unresponsiveness, not tolerance, was observed to *V. anguillarum* immersion vaccination before a critical weight of 0.4 g [23]. Tatner and Horne [24] also found that higher levels of ^{14}C-labelled *V. anguillarum* were taken up by direct immersion as the size of rainbow trout increased from 4.5 to 26.0 g.

In conclusion, mucosal FMS uptake and trafficking to the kidney and spleen increases with age in developing channel catfish, and appears to coincide with the development of acquired immunity at 3-wph as reported in the literature [6]. Particulate antigen exclusion may be attributed to a specific mechanism functioning to inhibit antigen access in channel catfish from hatch to around 3-wph (i.e., high mucus production, mucus composition), or, functional maturity of epithelial cells and or professional antigen-presenting cells in the form of uptake (receptors, phagocytosis) and trafficking (receptors, chemokines, and cytokines) may not be reached until around 3-wph. These results suggest that to maximise particulate antigen uptake and retention for productive acquired immune responses, channel catfish immersion vaccinations of particulate antigens should be administered at 3-wph or older.

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